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DRAWINGS ATTACHED

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(54) POLYSACCHARIDES AND THE PREPARATION THEREOF

(71) We, AJINOMOTO CO., INC., a corporation organised under the laws of Japan, of No. 7, 1-chome, Takara-cho, Chuo-ku, Tokyo, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to polysaccharides having anti-tumour properties and to processes for the preparation of the same.

It is well known that anti-tumour substances may be obtained by extracting various natural sources. Thus, for example, anti-tumour polysaccharides may be obtained by extracting the fruit body of Bacidiomycetes, e.g Grifola frondosa, Grifola gigantea or Fistulina hepatica of Polyporaceae; of bamboo leaves, bamboo grass, Graminaceae grass or yeast cells. However, the extracts are unsatisfactory since, although they may affect the cancer cells, they also damage normal tissues.

We have found that those polysaccharides which have been extracted from the fruit body of certain edible mushrooms show marked anti-tumour effects, that these polysaccharides are a mixture of an active anti-tumour glucan, inactive glucan and inactive heteroglycan, and that the active anti-tumour glucans can be easily isolated from the other constituents of the polysaccharide mixture by fractional dissolution and/or fractional precipitation.

It is known that glycans (which are also known as polysaccharides) may be homoglycans or heteroglycans. Homoglycans are polysaccharides which upon complete hydrolysis give only one species of monosaccharides; for example glucan (e.g. cellulose, starch, glycogen or dextran) consists of glucose units. Heteroglycans, however, are polysaccharides which on complete hydrolysis give at least two species of monosaccharides; for example, galactomannan will yield galactose and mannose.

In other words, glucan is a polymer of glucose.

According to one aspect of the present invention, there are provided two glucans obtained by extraction from an edible mushroom, and having anti-tumour properties.

One anti-tumour glucan is β-(1→3) glucan, the "β-" indicating that the glucan is a polymer of β-glucose (as opposed to α-glucose). In fact, this anti-tumour glucan is a linear long chain of glucose units each of which is joined by a β-glucoside link to the C_3 hydroxyl group of another glucose unit.

The other glucan which can have anti-tumour characteristics is β-(1→4)(1→6) glucan which consists of a main chain of glucose units joined by β(1→4) glucoside linkages and branches of glucose units joined by β-(1→6) glucoside linkages. An arrangement of this type can be found in amylopectin which is one of the two main constituents of starch. The anti-tumour activity of the anti-tumour β(1→4)(1→6) glucan according to the present invention is less than that of the β-(1→3) glucan of the invention. The infra-red absorption spectra of these two glucans are shown in the accompanying drawing, in which:—

Figure 1 is an infra-red absorption spectrum of the β-(1→3) glucan according to the present invention; and

Figure 2 is an infra-red absorption spectrum of the β-(1→4)(1→6) glucan according to the present invention.

The anti-tumour glucans according to the present invention are novel compounds

which are different in their biological effects and in their chemical and physiological properties from any of the known anti-tumour agents, especially in their non-toxicity.

According to another aspect of the present invention, there is provided a process for the preparation of a mixture of polysaccharides which includes at least one such glucan possesisng anti-tumour properties, which process comprises subjecting fruit body of an edible mushroom to extraction with hot water, concentrating the aqueous extract, and precipitating a mixture of polysaccharides, which includes at least one said anti-tumour glucan, by mixing the concentrated aqueous extract with a water-miscible organic solvent.

Preferably, the anti-tumour glucan will be separated from the other polysaccharides by, for example, the methods hereinafter described.

The present invention also includes said mixtures of polysaccharides when prepared by the foregoing process, and an anti-tumour glucan whenever separated from said mixture.

Edible mushrooms which can be used as the starting material in the process of the present invention include those belonging to the order Agaricales, e.g. *Lentinus edodes* (Berk) Sing, *Flammulina veltipes* (Court ex Fr.) Sing, *Tricholoma matsutake* (Ito et Imai) Sing, *Lyophyllum aggregatum* (Schaff ex. Secr.) Kuhner, *Pleurotus ostreatus* (Jacq ex Fr.) Quel, *Pleurotus spondoleucus* (Fr.) Quel, *Pholiota nameko* (T.Ito et Imai). The fruit body may be taken from wild or cultured mushrooms.

In order to purify the anti-tumour glucan according to the present invention, the following procedure may be adopted: namely, the fruit body of an edible mushroom is homogenised in water so as to produce an aqueous suspension. The homogenisation can be effected with a mixer or waring blender. Insoluble substances are then removed from resulting suspension, for example by filtering or centrifuging the suspension. The resulting aqueous layer is concentrated under reduced pressure, and at least one water-soluble organic solvent is added to the concentrated solution in order to precipitate the polysaccharides. The precipitation of polysaccharides from the extracted solution is preferably performed in two steps: firstly, an equivolume of an organic solvent is added to the concentrated solution in order to precipitate grey fibrous substance, which may be collected with a net, and then generally from two to five unit volumes of the organic solvent are added to obtain further precipitates, which may be collected by filtration or centrifuge. Examples of water-soluble organic solvents which can be used in the present invention include methanol, ethanol, propanol and acetone.

The precipitates of polysaccharides which are obtained are dissolved in an aqueous alkaline solution, and any protein which is included in the precipitates is removed by Sevag's method. The polysaccharides may then be purified by conventional methods, e.g. by salting out, by dialysis, by precipitation or by using an ion exchange resin.

The mixture of polysaccharides can be dried.

The anti-tumour polysaccharides may be used in the form in which they are obtained above, and consequently they are usually a mixture of active anti-tumour polysaccharides and inactive polysaccharides.

The polysaccharides mixture containing the active anti-tumour glucan, or the anti-tumour glucan *per se*, may be administered pharmaceutically by itself. More usually, however, they will be employed in a pharmaceutical composition in combination with a pharmaceutically acceptable carrier or diluent.

Also in accordance with the present invention, there is provided a process for separating the anti-tumour glucans from a mixture of active anti-tumour and inactive polysaccharides, which comprises dissolving said mixture in water, adding a strong basic compound so as to precipitate homoglycan salts of the strong basic compound, treating the precipitated homoglycan salts with different portions of an acidic solution of varying concentration in a manner such as to dissolve consecutively the different components of the homoglycan, whereby the mixture of polysaccharides is separated into its components, and recovering the active anti-tumour glucans.

In order to isolate each active anti-tumour glucan according to the present invention, a mixture of active anti-tumour and inactive polysaccharides which have been obtained by extraction from an edible mushroom is dissolved in a large amount of water, an aqueous solution of a strong basic compound is added in order to precipitate the salts of all the homoglycans with the basic compound. The precipitates are collected by filtration or centrifuge. Examples of strong basic compounds which can be used in the present invention include quaternary ammonium compounds, e.g. cetyltrimethyl ammonium hydroxide, cetyltrimethyl ammonium halide, and cetylpyridinium halide; and strong basic ion exchange resins. Only homoglycans react with the strong basic compounds to form precipitates, and the heteroglycans, which do not show any anti-tumour activity, and other impurities remain in the solution.

The isolated precipitates may be fractionated by treating them with acidic solutions of varying concentration. For example, the precipitates may be added to an acidic solution of low concentration, the mixture stirred, and glucan soluble in the acidic solution isolated from the water layer by adding organic solvents. Insoluble glucans which remained in the acidic solution may be collected by filtration, added to an acidic solution of higher concentration than that of the previous step, and glucan soluble in that acidic solution may be isolated by treatment in the same manner as in the previous step. Treatment similar to the above steps may be effected on the glucans insoluble in the prior acidic solutions, while increasing the concentration of acid, and glucan insoluble in all acid solutions is generally obtained in the last treatment. Acids used in the present invention are generally organic acids having a concentration of less than 50%, e.g. acetic acid or propionic acid, and mineral acids having a concentration of less than 0.3%. Each glucan fractionated by the procedure of the present invention is purified by Sevag's method and or precipitation, and determined for anti-tumour activities.

Another embodiment of the present invention provides a process for isolating the anti-tumour glucans from a mixture of active anti-tumour and inactive polysaccharides, obtained by the extraction of edible mushroms, which process comprises dissolving a mixture of active anti-tumour and inactive polysaccharides in water, and adding a strong basic compound so as to precipitate consecutively salts of the glucan with the strong basic compound. Each fraction of these precipitates can be separately treated with an acidic solution so as to produce the glucans, from which the active anti-tumour glucans can be recovered. The strong basic compound can be added in solid form or in the form of a solution.

The addition of the strong basic compound is continued until no further precipitation is observed (without any sharp elevation of the pH value). Each precipitate is collected, the precipitates are treated with acid to liberate the glucan, and the anti-tumour effect of each glucan is tested.

The present invention will now be illustrated by the following Examples.

Example 1

500 G. of the fresh fruit body of *Lentinus edodes* was washed, homogenised in 4 to 5 times its own volume of water with a waring blender, and boiled for 16 hours with stirring. Insoluble substances which remained were removed by centrifuging, one litre of the resultant supernatant liquid was concentrated under reduced pressure to one third of its initial volume, and the resulting concentrated solution was freeze-dried. The anti-tumour activity of the resulting powder was determined as follows:

Swiss albino mice each weighing about 20 g. were used for the anti-tumour assay. A 0.05 ml. dose of 7 day old Sarcoma 180 ascites was injected subcutaneously in the right groin of each mouse. A dose of the anti-tumour powder (corresponding to 200 mg. of the powder (suspended in distilled water) per kg. of mouse) was injected intraperitoneally daily for ten days starting 24 hours after tumour implantation. The tumour growth was measured weekly for five weeks, and compared with that of mice which had not been injected with the anti-tumour preparation. At the end of the fifth week, the mice were killed, the tumours were removed, and the inhibition ratios were determined from the weights of the tumours. The growth inhibition ratio was calculated from the following formula:

$$\text{Inhibition ratio (\%)} = \frac{A-B}{A} \times 100$$ wherein

A is the average tumour weight of the control group, and B is that of the powder treated group.

Complete regression, which is the number of mice completely cured out of 10 mice treated was also determined.

The following results were obtained:—

Dose (mg./kg./day)	*Inhibition ratio (%)*	*Complete regression*
200	80.7	6/10

Example 2

50 kg. aliquots of the fruit body of each of *Pleurotos ostreatus, Flammulina veltipes, Pleurotus spondoleucus, Tricholoma matsutake* and *Pholiota nameko* were each homogenised in 240 litres water with a mixer, concentrated by boiling for 20 hours to 120 litres, and filtered under pressure with heating. 80 Litres of the filtrate which was obtained were filtered in the process of Celite, and the resulting filtrate was freeze-dried to form a powder which was subjected to the test described in Example 1. The word "Celite" is a registered Trade Mark.

Results of anti-tumour test were as follows:

Samples extracted from	*Dose mg./kg./day*	*Inhibition ratio (%)*	*Complete regression*
Pleurotus ostreatus	200	75.3	5/10
Flammulina veltipes	200	81.1	3/10
Pleurotus spondoleucus	200	72.3	0/10
Tricholoma matsutake	200	91.8	5/9
Pholiota nameko	200	86.5	0/10

Example 3

Concentrated extract as obtained in Example 1 was poured onto 1.2 times its own volume of ethanol, and the grey fibrous precipitates (A) which formed were collected with a net, and washed with ethanol. 50 G. of the precipitate were homogenised in 2 litres of water with a waring blender for 5 minutes, 20 litres of water were added to the homogenised solution, and the resulting mixture was stirred to produce a clear solution. An aqueous 0.2*M* solution of cetyltrimethyl ammonium hydroxide was added dropwise to the 22 litre solution so as to form a colourless precipitate until no further precipitate was formed. The precipitate was collected by centrifuging, suspended and washed in ethanol, and added to 1.2 litres of 20% acetic acid. The resulting mixture was stirred for 5 minutes at room temperature, centrifuged, and fractionated into a precipitate (Friction I) and a supernatant solution (Fraction II). The supernatant was added to 4 times its own volume of ethanol and the precipitate which formed was collected by centrifuging and washed twice with ethanol and then with ether. The precipitates which were obtained were dried in vacuo, treated five times to remove protein by Sevag's method, and dissolved in 600 ml. water. 75 Ml. of 0.2*M* cetyltrimethyl ammonium hydroxide solution were added to the aqueous solution dropwise (pH 11.0), and the colourless precipitate which formed was collected by centrifuging. These precipitates were stirred into 300 ml. of 10% acetic acid, 600 ml. of ethanol were added to the resulting solution, and the precipitates which formed were collected by centrifuging.

These precipitates were washed with 100 ml. ethanol, and then twice with ether, and 2.2 g. of pure anti-tumour β-(1→4)(1→6) glucan (Fraction II-a) was obtained.

25 ml. of 0.2*M* cetyltrimethyl ammonium hydroxide were added (pH 12.5) to the supernatant which remained after the colourless precipitates were removed by centrifuging. Then, a small amount of NaCl was added, and the precipitate which formed (Fraction II-b) was treated in the same way as was Fraction II-a, so as to produce 4.5 g. of pure inactive α-(1→6) glucan (Fraction II-b).

To the supernatant liquid from which the precipitate of Fraction II-b was removed, 0.2*M* cetyltrimethylammonium hydroxide was added until the pH of the medium became 12.8. 5 times its own volume of ethanol was added and the precipitate which formed was collected and treated in the same way as above. 2.0 G. of pure inactive β-(1→6) glucan were obtained as Fraction II-c.

The precipitate which was Fraction I was added to 1 litre of 50% acetic acid, stirred for ten minutes in an ice bath and the solid matter was collected by centrifuging. The supernatant part did not contain any glucan. The solid part was washed with 500 ml. ethanol, dissolved in 2 litres of 6% NaOH solution, and any impurities were removed by centrifuging. Four litres of ethanol were added to the solution, and the precipitate which formed was collected and washed firstly with ethanol and then with ether. Protein included in the precipitate was removed by Sevag's method, and 12.3 g. of pure active anti-tumour β-(1→3) glucan were obtained.

The properties of the anti-tumour glucans which were obtained are as follows:

Fraction I: (β-(1→3) glucan)

(1) $a_D^{18} = +19.5 - 21.5°$ (C=1. *IN* NaOH)

(2) When fraction I was heated in a solution of acetic acid, acetic anhydride and sulphuric acid system, β-(1→3) oligosaccharides consisting of laminaribiose, laminaritriose and, laminaritetraose were detected by paper chromatography, thin layer chromatography and gas chromatography.

(3) A β-(1→3) oligosaccharide consisting of laminaribose was obtained by the partial hydrolysis of fraction I using dilute sulphuric acid, and glucose was the sole product upon complete hydrolysis.

(4) Glucose is quantitatively produced upon hydrolysis of fraction I using Screlotium exo-1, 3-β-glucanase.

(5) No mobility could be detected during electrophoresis with a sodium borate buffer solution of pH9.3 and only one spot was detected with alkali solution.

(6) The fraction I was slightly soluble in water, soluble in alkali solution, hot water and formic acid solution, and practically insoluble in organic solvents, e.g. ethanol, acetone or chloroform.

(7) The fraction I was easily decomposed at 70°C. in a 10% NaOH solution, but was rather stable in an acidic medium.

(8) Fraction I had a water content of 15 percent, as determined by differential thermal analysis.

(9) The fraction swelled and became a gel when dipped in water.

(10) Elementary analysis.

C(%) 40.54, H(%) 7.02, ash 0%

No other element, apart from oxygen, was detected.

(11) Molecular weight was 950,000 to 1,050,000, as as determined by the light scattering method.

Fraction II-a: β-(1→4)(1→6) glucan

(1) $a_D^{22} = -16.3°$ (C=1. H_2O)

(2) Glucose was the sole product of complete hydrolysis with a dilute acid.

(3) Only one spot was detected during electrophoresis with a sodium borate buffer

solution of pH 9.3, and with a 1*N* NaOH solution.

(4) The fraction was soluble in water, alkaline solution and formic acid solution.

(5) Cellobiose and gentiobiose were obtained on partial acetolysis.

(6) Erythritol, glycerol and glyceraldehyde were obtained on Smith's degradation.

(7) Elementary analysis.

C(%) 40.89, H(%) 6.72

No other element, apart from oxygen, was detected.

It was considered from the above results that the Fraction II-b was β-(1→4)(1→6) glucan.

Results of anti-tumour assays of glucans obtained in Example III, as well as of the starting material are shown in the following Table 1. The tests were conducted in the same manner as that described in Example 1.

Table 1

Sample	*Dose (mg./kg. x day)*	*Inhibition ratio (%)*	*Complete regression*
Starting material	20 x 10	69.5	2/10
	5 x 10	78.1	5/9
Fraction I	5 x 10	97.6	7/10
	1 x 10	100	10/10
	0.5 x 10	100	8/10
Fraction II-a	25 x 10	91.0	7/10
	5 x 10	94.0	6/10
	1 x 10	55.0	1/9
Fraction II-b	25 x 10	−37	0/10
	5 x 10	−18	0/10
	1 x 10	0	0/10
Fraction II-c	25 x 10	6.4	0/10
	5 x 10	−19.1	0/10

WHAT WE CLAIM IS:—

1. β-(1→3) glucan (as hereinbefore defined) obtainable by extraction from an edible mushroom, and having anti-tumour activity.

2. β-(1→4)(1→6) glucan (as hereinbefore defined) obtainable by extraction from an edible mushroom, and having anti-tumour activity.

3. Process for preparing a mixture of polysaccharides which includes at least one anti-tumour glucan, which process comprises subjecting fruit body of an edible mushroom to extraction with hot water, concentrating the aqueous extract, and precipitating a mixture of polysaccharides, which includes at least one said anti-tumour glucan, by mixing the concentrated aqueous extract with a water-miscible organic solvent.

4. Process according to Claim 3, wherein the mushroom is one belonging to the order *Agaricales*.

5. Process according to claim 4, wherein the mushroom belongs to the strain *Lentinus edodes*.

6. Process according to Claim 3, 4 or 5, wherein, prior to said concentration, the fruit body is homogenised in water so as to produce an aqueous suspension, and insoluble matter is removed from the suspension, the resulting aqueous layer then being concentrated, and wherein the precipitated polysaccharides are separated from the remainder of the mixture of the concentrated aqueous extract and said organic solvent, and proteinaceous impurities are removed from the separated polysaccharides by treating the same with an alkali.

7. Process according to claim 6, wherein the water-miscible organic solvent is methanol, ethanol, propanol or acetone.

8. Process according to claim 6 or 7, wherein protein present in the precipitated polysaccharides is separated using Sevag's method.

9. Process according to any one of claims 6 to 8, wherein the separate polysaccharides are purified by salting out, dialysis, precipitation or ion exchange.

10. Process according to any one of claims 3 to 9, which further comprises drying the mixture of polysaccharides.

11. A process according to claim 3 for preparing a mixture of polysaccharides which includes at least one anti-tumour glucan, substantially as described in either of the foregoing Examples 1 and 2.

12. A mixture of polysaccharides which includes at least one anti-tumour glucan, whenever prepared by the process claimed in any one of claims 3 to 11.

13. Process for separating an active anti-tumour glucan from a mixture of active anti-tumour glucan and inactive polysaccharides, obtained by hot water extraction from an edible mushroom, which process comprises dissolving the mixture in water, adding a basic compound to the resulting solution so as to precipitate the homoglycan salts of the basic compound, and treating the precipitated homoglycan salts with acidic solutions of increasing concentration so as to dissolve consecutively

the different components of the homoglycan, whereby the mixture of polysaccharides is separated into its components.

14. Process for separating an active anti-tumour glucan from a mixture of the active anti-tumour glucan and inactive polysaccharides, obtained by hot water extraction from an edible mushroom, which process comprises dissolving the mixture in water, adding portions of a solution of a basic compound to the solution of the mixture so as to cause the step-by-step precipitation of the components of the mixture, and collecting each separate precipitate, whereby the mixture of polysaccharides is separated into its components each of which is in the form of its basic salt.

15. Process according to claim 14, wherein the or each precipitate containing an anti-tumour glucan in the form of its basic salt is dissolved in an acidic solution to obtain the free glucan.

16. Process according to claim 13 or 15, which further comprises isolating the anti-tumour glucan from the acidic solution by mixing the latter with an organic solvent.

17. Process according to any one of claims 13 to 16, wherein the basic compound which is added to the resulting mixture is in the form of an aqueous solution.

18. Process according to any one of claims 13 to 16, wherein the basic compound which is added to the resulting mixture is in the form of a solid.

19. Process according to any one of claims 13 to 18, wherein the basic compound is a quaternary ammonium compound or a basic ion exchange resin.

20. Process according to claim 19, wherein the quaternary ammonium compound is a cetyltrimethyl ammonium halide, cetyltrimethyl ammonium hydroxide or a cetylpyridinium halide.

21. Process for separating an anti-tumour glucan from a mixture of an anti tumour glucan and inactive polysaccharides, substantially as described in the foregoing Example 3..

22. An anti-tumour glucan whenever separated from a mixture of an anti-tumour glucan and inactive polysaccharides by a process as claimed in any one of claims 13 to 21.

23. A pharmaceutical composition comprising, as an anti-tumour active ingredient, a mixture of polysaccharides as claimed in claim 12 or an anti-tumour glucan as claimed in claim 1, 2 or 22, and a pharmaceutically acceptable diluent or carrier therefor.

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1,279,104 COMPLETE SPECIFICATION
1 SHEET

FIG.1.

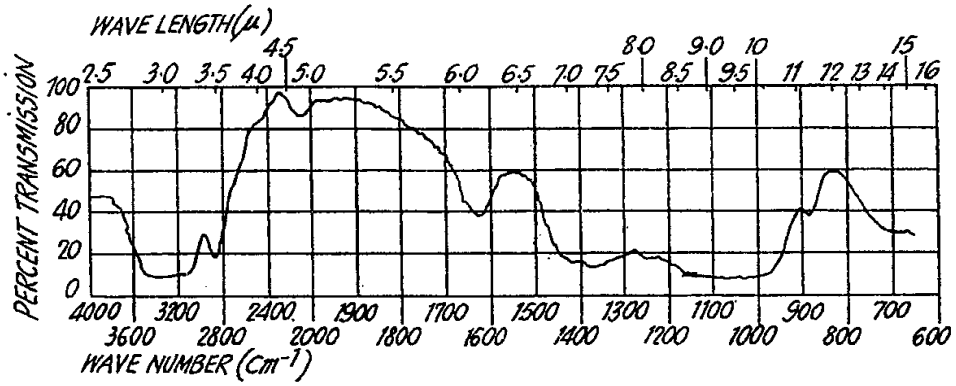

FIG.2.